

Epigallocatechin gallate inhibits the proliferation and induces apoptosis of multiple myeloma cells via inactivating EZH2

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Abstract. – **OBJECTIVE:** Epigallocatechin gallate (EGCG), the major chemical constituent of green tea, exhibits remarkable anti-tumor effect properties. In the present work, we aim to explore the effect and underlying mechanism of EGCG on multiple myeloma (MM) cells.

MATERIALS AND METHODS: The effects of EGCG on MM cells proliferation and apoptosis were determined by CCK-8 assay and flow cytometry assay. The siRNAs were used to inhibit endogenous expression of EZH2. Enforced expression of EZH2 in U266 cells was accomplished by transfecting EZH2 plasmid.

RESULTS: EGCG suppressed proliferation and induced apoptosis in U266 cells, which accompanied by EZH2 inhibition. Moreover, we revealed that enforced expression of EZH2 increased MM cells proliferation and reduced cell apoptosis, whereas EGCG partially reversed the effects of EZH2 on MM cells progression. In addition, qRT-PCR and Western blot showed that EZH2 overexpression increased Bcl-2 expression, and decreased BAX, BAK1 and cytochrome c expression in U266 cells exposed to EGCG.

CONCLUSIONS: Our data showed that EGCG inhibited MM cells proliferation and induced apoptosis by targeting EZH2 and modulated mitochondrial apoptosis pathway, indicating EGCG might act as an adjuvant for chemotherapy of MM patients.

Key Words:

Epigallocatechin Gallate, Multiple myeloma, Proliferation, Apoptosis, EZH2.

Introduction

Multiple myeloma (MM) is a malignant plasma-cell (PC) disorder and clinically defined when a PC neoplasm results in clinical complications¹.

MM accounts for approximately 1% of neoplastic diseases and 13% of all hematologic cancers². Despite remarkable improvements in treatment, MM remains an incurable disease³. Therefore, there is an urgent need to find effective drugs, which target tumor cells and do not harm normal cells.

Epigallocatechin gallate (EGCG, (-)-epigallocatechin-3-gallate) is an abundant polyphenol derived from green tea⁴. EGCG has been investigated as polyphenol for its potential application to human health and disease⁵. Several reports suggested the anti-cancer effect of EGCG in various cancer types both *in vitro* studies and animal models^{6,7}. Moreover, EGCG has been intensively studied as a chemo-preventive agent⁸. Increasing evidence showed that EGCG could act as a blocker for a series of signal transduction pathways related to carcinogenesis, such as cellular receptor-associated tyrosine kinase⁹.

Enhancer of zeste homolog 2 (EZH2) is a member of the Polycomb-group (PcG) family^{10,11}. PcG family members are involved in maintaining the transcriptionally repressive state of genes over successive cell generations¹². Recent studies suggested that EZH2 play critical roles in tumorigenesis¹³. For example, Yi et al¹⁴ showed that EZH2 mediated epigenetic silencing of TIMP2 promoted ovarian cancer migration and invasion. Yu et al¹⁵ found that miR-26a inhibited nasopharyngeal cancer cells invasion and metastasis by targeting EZH2. Li et al¹⁶ indicated that the degradation of EZH2 mediated by lncRNA ANCR attenuated the invasion and metastasis of breast cancer. Targeting EZH2 is now considered to be therapeutic strategies in cancer.

We investigated the effect and underlying mechanism of EGCG on MM cells. We determined the effects of EZH2 on EGCG induced cell

proliferation inhibition and apoptosis. We also tested the effects of EZH2 on key factor of mitochondrial apoptosis pathway. Taken together, our data suggested that EGCG might be a novel candidate as an adjuvant for chemotherapy of MM.

Materials and Methods

Cell and Chemicals

The human MM cell line U266 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). U266 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in a humidified atmosphere at 37°C with 5% CO₂. EGCG was purchased from Sigma-Aldrich (St. Louis, MO, USA) and used to treat U266 cells at 50 µM¹⁷.

Cell Transfection

SiRNA against EZH2 (si-EZH2), si-NC, pcDNA-EZH2, and empty vector pcDNA3.0 were purchased from GenePharma (Shanghai, China). The cells were transfected with si-EZH2 or pcDNA-EZH2 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Transfection efficiency was measured by qRT-PCR.

Cell Counting Kit-8 (CCK-8) Assays

Cell proliferation was analyzed by CCK-8 assay. In brief, cells were seeded in 96 well plates at a density of 1000 cells per well and cultured for 24 h, and then subjected to various treatments in each 4 replicate wells. At the indicated time points, each well was added with 10 µL CCK-8 reagent solution (Dojindo, Kumamoto, Japan), and incubated for 1 h. Optical density was determined by a microplate reader at the absorbance of 450 nm.

Cell Apoptosis Assays

Annexin V-FITC Detection Kit (Thermo Scientific, Waltham, MA, USA) was used for detecting cells apoptosis. After transfection, cells were incubated with Dulbecco's Modified Eagle Medium (DMEM) for 48 h. Then, the cells were collected and washed twice with phosphate-buffered saline (PBS). Annexin V-FITC (5 µL) and propidium iodide (PI) (10 µL) were added and the cells were incubated at 25°C for 15 min. The flow cy-

tometry (Beckman Coulter, Fullerton, CA, USA) was used for counting the stained cells and FACS-calibur was used for analyzing cell apoptosis.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to reversely transcribe RNA samples. For analysis of EZH2, a SYBR green mix PCR kit (TaKaRa, Tokyo, Japan) was used. The relative expression of EZH2 was determined by the 2^{-ΔΔCT} method normalized to GAPDH. The primers are as followed: EZH2 sense 5'-AGATGAAGCTGACAGAAGAGGGAA-3' and EZH2 anti-sense 5'-GCATAGCAGTTTGGATTTACCG-3'; GAPDH sense 5'-GTCAACGGATTTGGTCTGTATT-3' and anti-sense 5'-AGTCTTCTGGGTGGCAGTGAT-3'. The experiments were performed in triplicate.

Western Blot Assay

The total protein of cells was extracted using RIPA buffer supplemented with protease inhibitor PMSF (Roche, Basel, Switzerland). Western blot assay was performed as previously described¹⁸. The primary antibodies were obtained from Abcam (Cambridge, MA, USA). The intensity of protein bands was quantified using Quantity One software 4.5.0 basic (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

Statistical analysis was conducted using SPSS 20 (IBM, Armonk, NY, USA). Data were presented as mean ± standard deviation (SD). The quantitative data between groups was compared and analyzed by Student's *t*-test or one-way ANOVA. *p*<0.05 was statistically significant.

Results

EGCG Suppressed MM Cells Proliferation

Phytochemicals, due to their dietary origin, are presumed to be safer and well tolerated with relatively low toxicity than chemotherapeutic agent to human¹⁹. EGCG is one of such phytochemicals. Chemical structure of EGCG is provided in Figure 1A. We first explored the function of EGCG on MM cells proliferation and apoptosis. CCK-8 assay showed that the proliferation of U266 cells

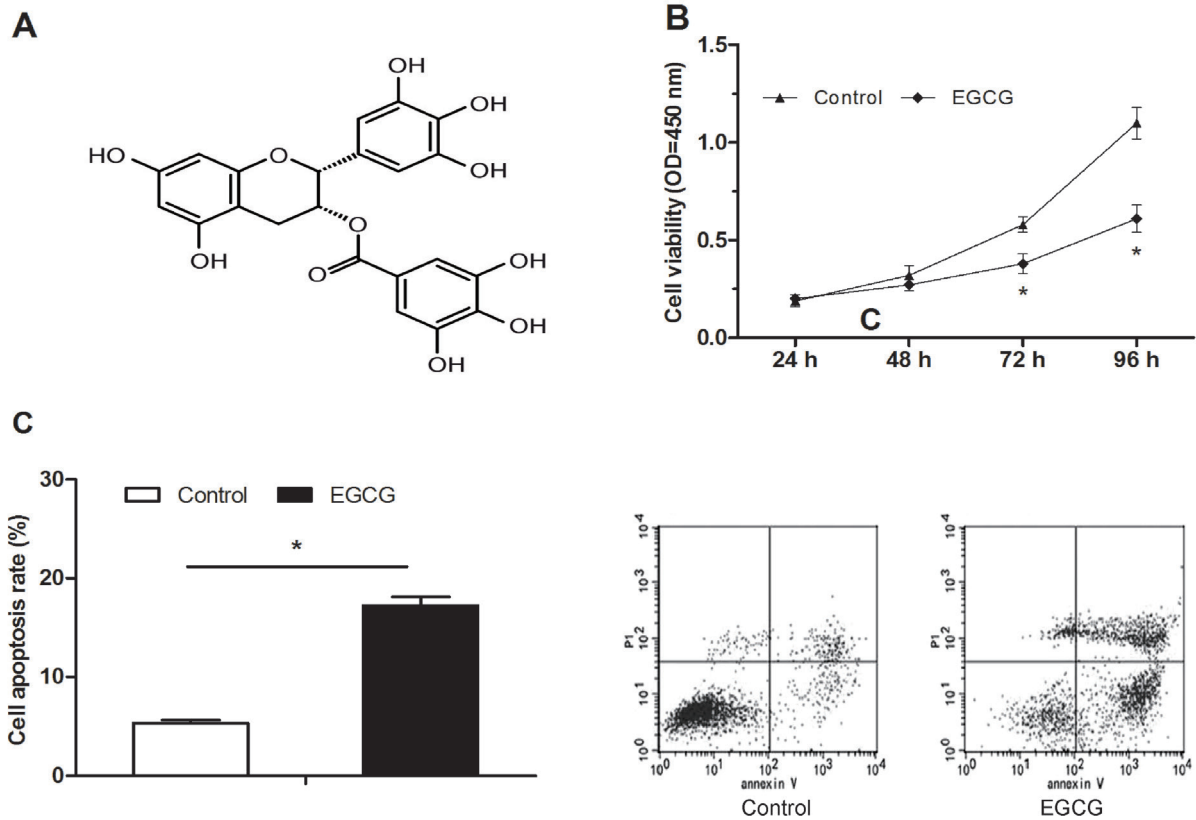


Figure 1. EGCG suppressed U266 cells proliferation. *A*, The chemical structure of EGCG. *B*, CCK-8 assay was used to determine the effect of EGCG on U266 cells. *C*, Cell apoptosis assay was used to explore the effect of EGCG on U266 cells. * $p < 0.05$.

treatment with EGCG was significantly inhibited compared to the control group (Figure 1B; $p < 0.05$). Cell apoptosis assay showed that EGCG could induce U266 cells apoptosis compared with the control group (Figure 1C; $p < 0.05$). These results indicated that EGCG could inhibit cell proliferation and induce apoptosis in MM cells.

EGCG Inhibited EZH2 Expression in MM Cells

We explored effects of EGCG on key regulator EZH2 in MM cells. U266 cells were exposed to EGCG. QRT-PCR and Western blot analysis showed that EGCG decreased the mRNA and protein expression of EZH2 in U266 cells compared to the control group (Figure 2A and 2B; $p < 0.05$).

EZH2 Modulated EGCG-Induced Cell Proliferation Inhibition and Apoptosis

Our previous study showed EZH2 was down-regulated in U266 cells exposed to EGCG, then EZH2 might play an important role in EGCG-induced proliferation inhibition and apoptosis. We determined the effects of EZH2 on cell prolifer-

ation, inhibition and apoptosis. Enforced expression of EZH2 was accomplished by transfecting pcDNA-EZH2 plasmid into U266 cells. The transfection efficiency was determined by qRT-PCR and Western blot assay (Figure 3A and 3B; $p < 0.05$). CCK-8 assay showed that EZH2 overexpression led to significantly promoted MM cells proliferation, simultaneous EGCG exposed completely reversed the promotion of cell proliferation (Figure 3C; $p < 0.05$). Cell apoptosis assay indicated that apoptosis was reduced in U266 cells transfected with pcDNA-EZH2, whereas EGCG partially reversed the reduction of cell apoptosis (Figure 3D; $p < 0.05$). Thus, these data indicated that EZH2 modulated EGCG induced cell proliferation inhibition and apoptosis.

The Effects of EZH2 on key Regulators of Mitochondrial Apoptosis

Furthermore, we speculated EZH2 involved in EGCG-induced apoptosis through the mitochondrial apoptosis pathway. In order to verify our speculation, we determined the effects of EZH2 on key regulators of mitochondrial apoptosis

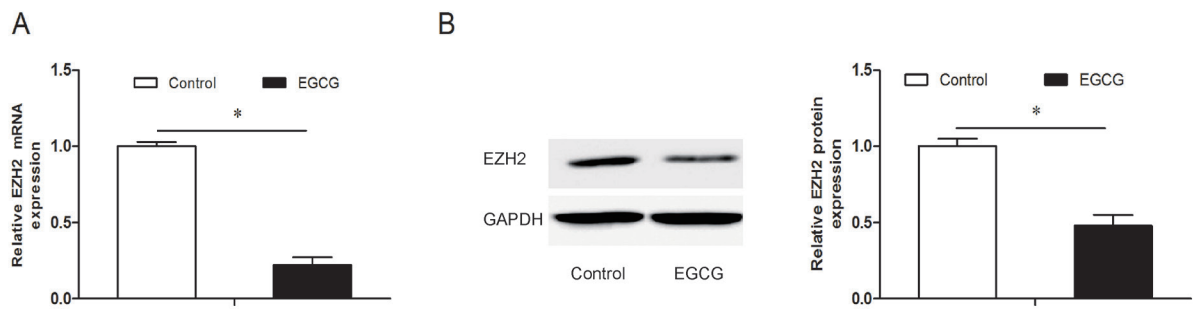


Figure 2. EGCG inhibited EZH2 expression in U266 cells. U266 cells were exposed to EGCG for 48 h. **A-B** The expression of EZH2 was determined by qRT-PCR and Western blot. * $p < 0.05$.

pathway. U266 cells were transfected with pcDNA-EZH2 or empty vector pcDNA3.0. QRT-PCR and Western blot analysis were performed to determine the expression of Bcl-2, BAX, BAK1 and cytochrome c (key factor of mitochondrial apoptosis pathway). Results showed that the expression of Bcl-2 was increased, while the expression of BAX, BAK1, and cytochrome c was decreased in U266 cells transfected with pcDNA-EZH2 (Figure 4A and 4B; $p < 0.05$). These data indicated that EZH2 probably function as an inhibitor of mitochondrial apoptosis pathway during EGCG-induced MM cells apoptosis.

Discussion

In recent years, researchers demonstrated an interest in the prospects of phytochemicals, natural anticancer agents from plants, due to the multitude of effects of these agents on cancer related molecular signaling pathways, which showed no or minimal toxicity for normal cells²⁰. EGCG, an antioxidant polyphenol flavonoid isolated from green tea, has been identified to serve a chemo preventive role by certain studies⁴. Recent studies showed that EGCG play important roles in tumor progression. For example, Lai et al²¹ found that EGCG could modulate cell proliferation in human pancreatic cancer cells and rat osteosarcoma cells *in vitro*. Modernelli et al²² indicated that EGCG antagonized Bortezomib cytotoxicity

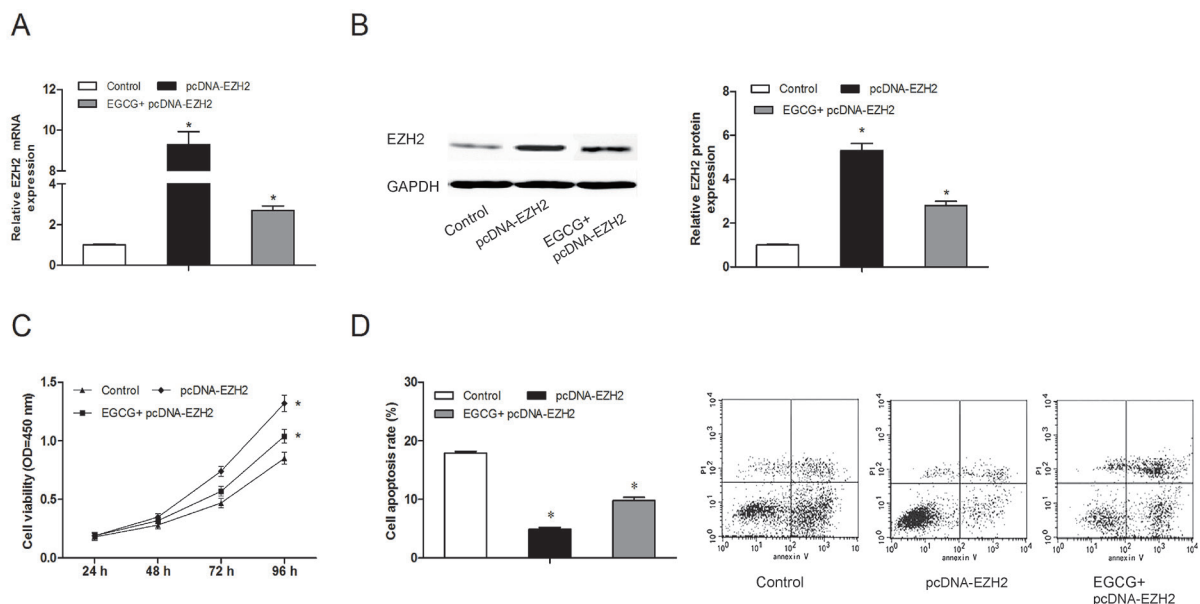


Figure 3. The effects of EZH2 on EGCG induced U266 cells proliferation inhibition and apoptosis. **A-B** The expression of EZH2 in U266 cells treated with pcDNA-EZH2, or EGCG+pcDNA-EZH2 was determined by using qRT-PCR and Western blot analysis. **C**, Cell proliferation in U266 cells treated with pcDNA-EZH2, or EGCG+pcDNA-EZH2. **D**, Cell apoptosis in U266 cells treated with pcDNA-EZH2, or EGCG+pcDNA-EZH2. * $p < 0.05$.

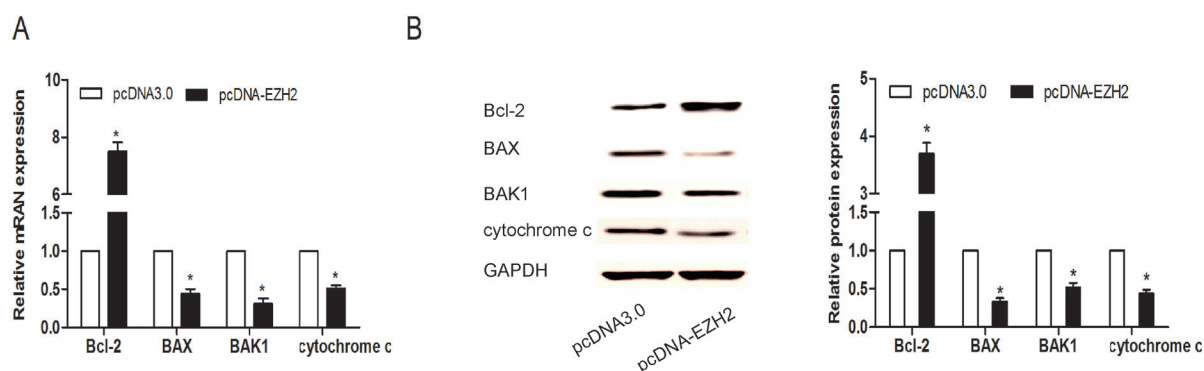


Figure 4. The effects of EZH2 on key regulators of mitochondrial apoptosis pathway. *A-B* QRT-PCR and Western blot were performed to determine the expression of Bcl-2, BAX, BAK1, and cytochrome c in U266 cells transfected with pcDNA-EZH2 or control vectors. * $p < 0.05$.

in prostate cancer cells by an autophagic mechanism. Cerezo-Guisado et al²³ showed that EGCG activation of Akt, ERK1/2, and p38d enhances HT-29 cell death. However, the function and the underlying mechanism of EGCG on MM cells remain unclear.

Our data showed that EGCG suppressed MM cells proliferation and induced apoptosis. Decreased expression of EZH2 was observed in U266 cells after they were exposed to EGCG, indicating that EZH2 might play important roles in the effects of EGCG on MM. The function studies showed that enforced expression of EZH2 promoted MM cells proliferation and reduced cell apoptosis, whereas EGCG partially reversed the effects of EZH2 on MM cells progression. These data indicated that EZH2 protected MM cells from EGCG-induced growth, inhibition and apoptosis. EZH2 is a negative regulator in inhibitory effects of EGCG on MM.

We further explored the role of EZH2 on key members of mitochondrial apoptosis pathway. Intrinsic control of apoptosis requires activation of cytosolic caspases by mitochondrial cytochrome c release and involves regulation of mitochondrial outer membrane permeabilization by Bcl-2 family proteins²⁴. Bcl-2 proteins promote cell death including those containing either multiple BH domains (Bax, Bak) or a single BH3 sequence (Bad, Bik, Bid, Puma, Bim, Bmf, and Noxa)²⁵. Pro-survival, multiple BH domain Bcl-2 proteins include Bcl-2, Bcl-xL, and Bcl-w²⁶. Pro-apoptotic Bax and Bak oligomerize at the mitochondria to alter membrane permeability and allow cytochrome c release into the cytosol^{27,28}. We showed that EZH2 increased accumulation of Bcl-2 and decreased expression of Bax and Bak, a cytochrome c in

U266 cells. These results indicated that EZH2 probably function as an inhibitor of mitochondrial apoptosis pathway during EGCG-induced apoptosis.

Conclusions

We demonstrated that EGCG could inhibit MM cells growth and induce apoptosis. Moreover, EGCG might suppress cell proliferation through EZH2 dependent mitochondrial apoptosis pathway. Taken together, our study not only provided a new insight into MM treatment, but also a new mechanism of EGCG on the anti-proliferation roles in tumors.

Acknowledgments

No.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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